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(54) Title: QUIESCENT CELL DIPEPTIDYL PEPTIDASE: A NOVEL CYTOPLASMIC SERINE PROTEASE

(57) Abstract: Disclosed herein are the cDNA and polypeptide sequences of a novel cytoplasmic post-prolyl dipeptidase, QPP. QPP is expressed in T-cells and in neurons, and functions to protect quiescent cells from apoptotic death. Therefore, QPP can be used as a therapeutic to inhibit apoptotic cell death and as a target in screening for compounds that modulate cell death.

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QUIESCENT CELL DIPEPTIDYL PEPTIDASE:
A NOVEL CYTOPLASMIC SERINE PROTEASE

Background of the Invention

10 This invention relates to a novel cytoplasmic post-prolyl dipeptidase, and its role in regulating apoptosis, or programmed cell death (PCD).

Apoptosis is a physiological form of cell death, and it shapes a number of diverse biological processes, such as development and homeostasis. It occurs in response to diverse stimuli which fall into two categories. The first is activation-induced cell death following specific stimulation; and the second is death by neglect after withdrawal of a life-promoting stimulation, such as a growth factor, serum or cell-cell contact. While these two types of PCD take place under very different circumstances, both depend on the activation of caspases, a family of cysteine proteases which are present in the cytoplasm of cells as inactive proenzymes.

20 Apoptotic stimuli lead to the activation of certain caspases by specific proteolytic cleavage, enabling them to activate other caspases through a proteolytic cascade, which eventually leads to cell death. Studies of activation-induced cell death through the Fas/TNF receptors have implicated the death effector domain-containing protease FLICE (caspase 8) in the initiation of the caspase cascade. 25 However, while most cells contain all the components of the apoptotic machinery,

and are susceptible to PCD by neglect, no caspase-cascade regulator induced under these conditions, has been identified.

With respect to apoptosis in the nervous system, recent studies have shown that neuronal cell death resulting from degeneration or trauma occurs by the process of apoptosis, the biochemical hallmarks of which include cytolemmal membrane blebbing, cell soma shrinkage, chromatin condensation, and DNA laddering. Thus, given the role of apoptosis in neural degeneration, it would be highly desirable to have a process for identifying compounds that modulate this apoptosis. Such compounds could be effective as therapeutic agents in the treatment or prevention of neural conditions associated with inappropriate neural degeneration. However, one of the fundamental technological problems associated with the identification of these compounds is the lack of suitable targets to use in screening for regulators of apoptosis. Thus, there is a need to identify new enzymatic targets as a first step in developing new therapeutic compounds.

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Summary of the Invention

We have discovered a novel, cytoplasmic post-prolyl dipeptidase, which has similarities to, but is distinct from, the membrane-bound T-cell serine protease CD26. This enzyme is expressed in quiescent, non-cycling T-cells and in neurons, and is called quiescent cell post-prolyl dipeptidase (QPP; previously termed 20 DPIVb).

In a first aspect, the invention features a substantially pure, recombinant quiescent cell post-prolyl dipeptidase polypeptide (QPP), wherein the polypeptide inhibits quiescent cells from undergoing apoptosis. Preferably, the polypeptide comprises the amino acid sequence of SEQ ID NO 6.

In a second and related aspect, the invention features a substantially pure nucleic acid molecule encoding a QPP polypeptide, wherein the nucleic acid sequence comprises SEQ ID NO 5, or a sequence that is complementary to a sequence that hybridizes to SEQ ID NO 5 under stringent hybridization conditions
5 encoding a QPP polypeptide, or encodes a polypeptide of SEQ ID NO 6.

The invention also features a host cell, for example, yeast, E. coli, Chinese hamster ovary (CHO), or fibroblast, transformed or transfected with a transgene that includes a nucleic acid molecule encoding a QPP polypeptide, wherein the nucleic acid molecule is operably linked to a regulatory sequence, and
10 wherein the nucleic acid sequence comprises SEQ ID NO 5, or a sequence that is complementary to a sequence that hybridizes to SEQ ID NO 5 under stringent hybridization conditions, or encodes a polypeptide of SEQ ID NO 6.

In a related aspect, the invention features a method of producing a QPP polypeptide including transforming or transfecting a cell with a nucleic acid
15 molecule encoding a QPP polypeptide, wherein the nucleic acid molecule is operably linked to a regulatory sequence, and further wherein the nucleic acid sequence comprises SEQ ID NO 5, or a sequence that is complementary to a sequence that hybridizes to SEQ ID NO 5 under stringent hybridization conditions, or encodes a polypeptide of SEQ ID NO 6; culturing the cell under conditions for
20 expressing the nucleic acid; and isolating the QPP polypeptide.

The purified QPP sequences of the invention have a number of utilities. The invention features methods for: 1) identifying a therapeutic candidate compound that modulates a QPP effect in quiescent cells, which includes contacting a recombinant QPP polypeptide, preferably, the QPP polypeptide of
25 SEQ ID NO 6, or a transformed or transfected cell encoding and expressing a

recombinant QPP polypeptide, with the compound and measuring QPP biological activity; 2) inhibiting apoptosis in a quiescent cell, which includes contacting the cell with a recombinant QPP polypeptide, preferably, the QPP polypeptide of SEQ ID NO 6, in an amount sufficient to protect the cell from death when an apoptosis stimulus is administered; and 3) treating a mammal for a condition related to quiescent cell death, that includes administering to the mammal a recombinant QPP polypeptide, preferably, the QPP polypeptide of SEQ ID NO 6, in an amount sufficient to inhibit the cell death. Preferably, the condition is a neurodegenerative disorder or an immune system disorder, and the QPP polypeptide may be

5 administered by transplanting a host cell into the mammal, wherein the host cell is *ex vivo* transfected with a transgene comprising a nucleic acid molecule encoding a QPP polypeptide, wherein the nucleic acid molecule is operably linked to a regulatory sequence and is expressed in the transplanted host cell.

10 10 administered by transplanting a host cell into the mammal, wherein the host cell is *ex vivo* transfected with a transgene comprising a nucleic acid molecule encoding a QPP polypeptide, wherein the nucleic acid molecule is operably linked to a regulatory sequence and is expressed in the transplanted host cell.

The recombinant QPP sequences can also be used to make antibodies

15 15 (polyclonal, monoclonal, or recombinant) using conventional methods, involving immunization of, e.g., rabbits, mice, or human volunteers. The antibodies can be used in standard ELISA assays to measure QPP levels in patients being tested for diseases which potentially involve increased or decreased QPP levels; for example, HIV patients, who have lost QPP-containing T-cells, will exhibit decreased QPP

20 20 levels, with the QPP concentration being diagnostic of the stage of the disease. Generally, because QPP is a cytoplasmic enzyme, the assay is carried out on peripheral blood lymphocyte samples which have first been treated to lyse T-cells to release the enzyme.

By "substantially pure" is meant a polypeptide or nucleic acid that has

25 25 been separated from components which normally accompany it, or which

accompany it upon recumbent expression. An enzyme is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably, at least 90%, and most preferably, at least 99%, pure
5 QPP by weight. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "apoptosis" is meant the process of programmed cell death wherein a dying cell displays a set of well-characterized biochemical hallmarks which include cytolemmal membrane blebbing, cell soma shrinkage, chromatin condensation, and DNA laddering.
10

By "stringent hybridization conditions" is meant incubation conditions that destabilize mismatched heteroduplexes. Conditions for high stringency hybridization, such as for PCR, Northern, Southern, *in situ* hybridization, or DNA sequencing, are described, for example, in F. Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1994.
15

By "transformed" or "transfected" cell is meant a cell in which foreign nucleic acid molecules have been introduced. Lipofection, DEAE-dextran-mediated transfection, microinjection, protoplast fusion, calcium phosphate precipitation, retroviral delivery, electroporation, and biolistic transformation are
20 just a few of the methods known to those skilled in the art which may be used. For example, biolistic transformation is a method for introducing foreign molecules into a cell using velocity driven microprojectiles such as tungsten or gold particles. Such velocity-driven methods originate from pressure bursts which include, but are not limited to, helium-driven, air-driven, and gunpowder-driven techniques.
25 Biolistic transformation may be applied to the transformation or transfection of a

wide variety of cell types and intact tissues including, without limitation, intracellular organelles (e.g., and mitochondria and chloroplasts), bacteria, yeast, fungi, algae, animal tissue, and cultured cells.

- By "apoptotic stimulus" is meant any chemical or physical treatment 5 which initiates apoptosis as defined above. For example, nerve growth factor withdrawal, hypoxia, exposure to staurosporine, and cerebral ischemia are stimuli capable of inducing apoptosis in neurons.

The "quiescent cell" is meant a non-cycling cell in a non-replicative or resting state.

- 10 By "QPP effect" is meant QPP's VbP-sensitive protection against apoptosis in quiescent cells.

- By "test compound" is meant a chemical, be it naturally-occurring or artificially-derived, that is surveyed for its ability to modulate cell death, by employing one of the assay methods described herein. Test compounds may 15 include, for example, small molecules, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

- By "therapeutic candidate compound" is meant a compound that modulates a QPP effect, and is, therefore, a candidate for use as a therapeutic to 20 inhibit cell death in quiescent cells.

- By "measuring QPP biological activity" is meant analyzing the effect of a test compound, whether the effects be chemical or physical. The material being analyzed may be an animal, a cell, a lysate or extract derived from a cell, or a molecule derived from a cell. The analysis may be for the purpose of detecting a 25 change in QPP biology such as altered RNA stability, altered protein stability,

altered protein levels, altered enzymatic activity, or an altered rate of QPP-mediated apoptotic death. The means for analyzing may include, for example, antibody labeling, immunoprecipitation, substrate cleavage assays, and methods known to those skilled in the art for detecting nucleic acids.

5 By "modulates" is meant changing, either by decrease or increase.

By "operably linked" is meant that a gene and a regulatory sequence are connected in such a way as to permit expression of the gene product under the control of the regulatory sequence.

10 By a "transgene" is meant a nucleic acid sequence which is inserted by artifice into a cell and becomes a part of the genome of that cell and its progeny. Such a transgene may be partly or entirely heterologous to the cell.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Description of the Figures

15 Figure 1 shows the amino acid sequences of four peptide fragments of QPP following tryptic digestion (SEQ ID NOs 1-4).

Figure 2 shows the QPP cDNA sequence (SEQ ID NO 5) and amino acid sequence (SEQ ID NO 6).

Detailed Description

20 We have discovered that a novel polypeptide, QPP, isolated from T-cells in healthy individuals, is involved in the protection of T-cells from apoptosis. This cell death-related property of QPP was discovered during studies of T-cell survival during HIV infection. Our observations led us to hypothesize that the inhibition of

QPP in HIV-infected individuals caused resistance to full T-cell activation, which further prevents differentiation into effector cells, and eventually leads to T-cell death. When not inhibited, QPP prevents this apoptosis in resting T-cells by blocking the catalytic activation of caspases. QPP's dipeptidase activity is present 5 in the cytoplasm of a number of types of normal, resting human T-cells, e.g., CD4 cells and Jurkat cells. In addition to T-cells, QPP is also expressed in neurons.

QPP Discovery and Purification

The work leading up to the discovery of QPP began with the question of whether, in resting cells, the apoptotic machinery may be kept inactive by the basal 10 activity of cellular enzymes. To find an answer, various enzymatic inhibitors were screened for their ability to trigger PCD in overnight cultures of freshly isolated peripheral blood mononuclear cells (PBMC).

1. VbP Causes Cell Death in Resting T Cells

We observed a striking increase in the number of dead cells in cultures 15 containing the L-isomer of Val-boroPro (VbP), an inhibitor of dipeptidyl peptidase IV (DPPIV), compared to cultures containing media alone or the inactive D-isomer of the inhibitor, d-Val-d-boroPro--a toxicity control. Dead cells were apparent as early as 4 hours after the addition of the L-isomer of VbP, with maximal death occurring within 24 hours (about 70%). When subpopulations of PBMC were 20 tested for susceptibility to VbP-induced death, we observed that CD19⁺ B cells and CD11b⁺ monocytes were resistant, while purified T-cells (CD4⁺/CD8⁺) showed greater sensitivity than whole PBMC. These results imply that the molecular target of VbP plays a role in T-cell survival. Surprisingly, activated PBMC are resistant to VbP-induced PCD, but regain sensitivity if maintained in

culture until the cells return to a non-cycling phase (G_0).

2. Mechanisms of VbP-induced Death

We first demonstrated that the death induced by VbP in resting T cells was caspase-dependent apoptosis and was independent of Fas and the proteosome 5 pathway.

First, turning to caspase-mediated apoptosis, the appearance of phosphatidylserine (PS) on the outer leaflet of the plasma membrane is a caspase dependent characteristic of apoptotic cells. By using the PS binding protein FITC-annexin V, we detected a time-dependent increase in the amount of PS on the 10 surface of cells treated with VbP, establishing that VbP induces apoptosis. The role of caspase in VbP-mediated PCD was confirmed, given that the general caspase inhibitor Z-VADfmk blocked VbP-mediated T-cell death.

To determine if other known mediators of activation-induced death were involved, we investigated Fas, the well defined and prototypic activation-induced 15 death pathway of T-cells, and the proteosome pathway. Fas was studied using anti-Fas mAb M3, which prevents PCD by blocking Fas-FasL interactions. The proteosome pathway was investigated using lactacystin, an inhibitor of proteosome activity, which induces cell death through the disregulation of proteins necessary for cell cycle progression, and does not induce PCD in resting PBMD. Although 20 these pathways typically affect activated cells, and PBMC are predominantly resting cells, we hypothesized that exposure to small molecule drugs like VbP might induce the expression of Fas ligand (FasL) or have other unanticipated effects that activate these pathways. However, neither the anti-Fas mAb M3 nor lactacystin had any effect on VbP-mediated death in non-cycling T-cells.
25 (However, after activation, PBMC are susceptible to lactacystin-induced death.)

These findings confirm that VbP- mediated PCD in resting cells proceeds through the caspase cascade and is independent of Fas-FasL interactions or a proteosome-mediated cell cycle block.

3. The VbP Target, QPP, Is Pharmacologically Distinct From DPIV

5 VbP was designed to inhibit DPIV, a catalytic activity attributed to the surface memory T-cell antigen CD26. However, VbP has an additional target, given that VbP caused T-cell death to the same degree in both CD26⁺ and CD26⁻ resting T-cell subpopulations. Since VbP is a highly potent inhibitor of DPIV, the novel VbP target is an enzyme with substrate specificity similar to that of DPIV.

10 Furthermore, peptidyl boronic acids, like VbP, are effective inhibitors of serine proteases, but do not inhibit cysteine proteases, indicating that the cytosolic enzyme responsible for AlaProAFC cleavage belongs to the serine class of proteases. Thus, we began to biochemically characterize the novel target by assaying cleavage of the DPIV substrate AlaProAFC.

15 First, membrane and cytosolic fractions were prepared from freshly isolated PBMC. The cytosolic preparation (110,000 g supernatant) contained a pH dependent AlaProAFC cleaving enzymatic activity. This activity is not the result of DPIV action, because CD26 (DPIV) fractionates with the membranes (110,000 g pellet). Optimal AlaProAFC cleavage occurred at pH 7.5, excluding lysosomal proteases as the source of this activity. The enzymatic activity was inhibited by VbP and was partially inhibited by high concentrations of benzamidine and the serine/cysteine protease active site titrants PMSF, TLCK, NEM and IAA, which most likely act as nonspecific alkylating agents at these high concentrations.

20 Because of the similar substrate specificities of the new cytoplasmic peptidase QPP and the known enzyme DPIV, we compared the inhibitory profile

of QPP to DPIV. Several known DPIV peptidase inhibitors were tested on QPP: 1) VbP; 2) the fluoroolefin containing (Z)-Ala-[CF=C]-Pro-NHO-Bz(4NO₂) (L-125); and 3) NO₂-Z-Lys-thiazolidide (Lys-thiazolidide). In addition, another compound related to Lys-thiazolidide, NO₂-Z-Lys-piperidine (Lys-piperidine), 5 which does not inhibit DPIV, was also tested. QPP inhibition was achieved with similar concentrations of VbP and Lys-thiazolidide, the most potent inhibitors of this activity. The related compound, Lys-piperidine, did inhibit QPP at higher concentrations than Lys-thiazolidide, and the DPIV inhibitor, L-125, did not inhibit QPP. Therefore, the differences in the inhibitory profiles of DPIV and the 10 quiescent T-cell VbP target (QPP) verified that QPP is distinct from DPIV. Furthermore, QPP's role in the VbP-induced T-cell death was confirmed by the fact that the inhibitors' effects on QPP's cleaving activity closely correlated with the inhibitors' effects on PCD induction in PBMC cultures.

Given our functional and pharmacological data, QPP is established as a 15 novel enzymatic inhibitor of the caspase cascade in quiescent T-cells, and is a target for the regulation of this pathway. In resting T-cells, the caspase cascade is kept inactive through the action of QPP; inhibition of QPP allows activation of the caspase cascade, leading to cell death. The implication of this model, that resting T cells are poised to die, is consistent with the notion that PCD is a constitutive 20 program which must be suppressed. Resting T-cells seem to be the sole target of VbP- induced death in the PBMC population, despite the presence of similar peptidase activities in the cytoplasm of activated T-cells and other blood cells. Interestingly, we observed a 9-fold reduction of AlaProAFC specific cleaving 25 activity in cytoplasmic extracts from PHA activated T-cell blasts compared to that of quiescent PBMC, suggesting that this activity is replaced by other inducible

proteolytic activities upon cellular activation. Thus, we anticipate that QPP is the first identified member of a family of proteolytic activities which regulate the caspase cascade in quiescent cells.

Methods. Human PBMC were isolated from the blood of healthy donors by centrifugation on a Ficoll-Hypaque gradient using standard techniques. T-cells and monocytes were isolated by sorting on a Becton Dickinson FACStar plus flow cytometer using biotinyl-anti-CD4 plus biotinyl-anti-CD8 (Leu2a plus Leu2b, Becton Dickinson) or anti-CD11b (44-biotin, Sigma), and phycoerythrin streptavidin, CD26⁺ T cells were isolated by sorting with the anti-CD26 mAb 1F7 (C. Morimoto, Dana-Farber Cancer Inst.). B cells were isolated by selection with biotinyl-anti-CD19 mAb (D. Thorley Lawson, Tufts Univ.) And MACS microbeads (Miltenyl Biotec). Sorted cell populations were >90% pure. Cells were washed several times in PBS and resuspended in serum free AIM V medium (Gibco), and plated at 1-2 x 10⁵ cells/well in 96 well flat-bottom plates. Enzyme inhibitors and mAb were added and the cells incubated for 18-24 hours in 5% CO₂ at 37 C. Cellular death was quantitated by staining the cells with 20 g/ml 7-amino actinomycin D (Sigma), hypotonic propidium iodide (Sigma), or FITC-annexin V (Southern Biotech Associates). Activated cells (PHA Blasts) were stimulated for 3 days with 5 g/ml phytohemagglutinin (PHA, Sigma). Long Term Cultured cells were cultured with PHA for 3 days, washed several times and cultured an additional 32 days without stimulating agents. Additional reagents: anti-FAS mAb M3 (D. Lynch, Immunex); Z-VADfmk (Enzyme System Products); lactacystin (E.J. Corey, Harvard Univ.); VbP, Lys-piperidine, Lys-thiazolidide (R. Snow and A. Kabcenell, Boehringer Ingelheim Pharmaceuticals), L-125 (J. Welch, State Univ. of N.Y., Albany).

- Enzyme Characterization. Human PBMC (430×10^6 cells) were isolated from 450 ml whole blood. Cells were resuspended in 7 ml ice cold lysis buffer (0.02 M Tris pH 7.8, 4 g/ml aprotinin, 8 g/ml leupeptin, 8 g/ml antipain) and held on ice 10 min before homogenization by 10 strokes of a Dounce homogenizer. EDTA was added to a final concentration of 5mM, and the homogenate centrifuged at 1000 g for 10 min. at 4 C. The resulting supernatant was centrifuged at 45,000 g for 20 min. at 4 C. The 45,000 g supernatant was centrifuged at 110,000 g for 1 hour at 4 C, the 110,000 g supernatant was used as soluble cytoplasmic extract. The 45K and 110K pellets were combined, resuspended in 10 ml lysis buffer, and centrifuged at 110,000 g for 20 min. at 4 C. The resulting pellets were combined and resuspended in lysis buffer/1% Triton X-100 and used as the membrane preparation. Peptidase activity was measured by monitoring the 1 min. accumulation of the fluorescent product 7-amino-4-trifluoromethylcoumarin (AFC) liberated from the substrate AlaProAFC (Enzyme Systems Products), using a Perkin-Elmer fluorescence spectrophotometer (excitation 400 nm, emission 505 nm). The optimal pH for AlaProAFC cleavage was determined using: 0.05 M acetate pH 4.5, 5.0; 0.2 M piperazine pH 5.5, 0.05 M NaPhosphate pH 6.0, 6.5, 7.0; 0.05 M HEPES pH 7.5, 8.0, 0.02 M TRIS pH 8.5, 9.0; 0.05 M HEPES pH 7.5 was used for all subsequent enzymatic assays.
- 15 Systems Products), using a Perkin-Elmer fluorescence spectrophotometer (excitation 400 nm, emission 505 nm). The optimal pH for AlaProAFC cleavage was determined using: 0.05 M acetate pH 4.5, 5.0; 0.2 M piperazine pH 5.5, 0.05 M NaPhosphate pH 6.0, 6.5, 7.0; 0.05 M HEPES pH 7.5, 8.0, 0.02 M TRIS pH 8.5, 9.0; 0.05 M HEPES pH 7.5 was used for all subsequent enzymatic assays.
- 20 The general protease inhibitors: PMSF, benzamidine, TLCK, aprotinin, leupeptin, IAA and NEM were purchased from Sigma. All reported catalytic rates are the average of 3 independent determinations.

We detected an enzymatic activity (cleavage of the substrate AlaProAFC) in the soluble fraction of CD26-Jurkat cells. The activity is inhibited 25 by the DPIV inhibitors XaaboroPro (Bachovchin et al. U.S. Patent No. 5,462,928,

hereby incorporated by reference). However, the activity is distinct from DPIV based upon differential sensitivity to other inhibitors (thiazolidide, piperidide, PMSF). The activity is greatly reduced (more than 50%) at pH 5.5.

5 QPP Purification. We purified QPP from Jurkat cytoplasm. The following general scheme produces a 1000-fold purification of QPP with a 27% yield:

- 10 1. Jurkat cytoplasm (110,000 g supernatant);
 2. Acid soluble fraction (0.05 M acetate pH 4.5);
 3. Cation Exchange (SP); and
 4. Gel filtration (Superdex 12).

In more detail, the above-outlined purification scheme is carried out as follows. First, Jurkat cells (10^6 - 10^{11} cells) are grown and a cell pellet is obtained by centrifugation. The cell pellet is stored in frozen condition.

15 The frozen pellet is thawed by the addition of ice cold lysis buffer, in the amount of approximately 1 ml per 10^8 cells. The liquified material is homogenized with ten strokes of a Dounce homogenizer, and then clarified by centrifugation at 1500 g. The supernatant is removed, and reserved. The 1500 g pellet is resuspended in lysis buffer and homogenized with ten strokes of a Dounce homogenizer. Clarification is again carried out by centrifugation at 1500 g at 4 C.

20 The 1500 g supernatants are then combined, and EDTA is added to 5 mM. The resultant liquid is centrifuged at 75,000 g at 4 C for twenty minutes, and the supernatant is then removed and centrifuged at 110,000 g at 4 C, for 60 minutes. The resultant supernatant, containing the cytosolic extract, is the QPP activity-containing fraction on which the subsequent purification steps are carried
25 out.

This procedure does not yield a homogeneous preparation, and attempts at further purification (ion exchange, affinity chromatography) resulted in the loss of nearly all protein in the sample, and recovery of very little activity.

To identify the component of this purified preparation responsible for XaaPro-cleaving activity, we established that diisopropylfluorophosphate (DFP) inhibits the activity in the SP purified fraction. An aliquot of the most pure preparation was then labeled with 3H-DFP and separated by SDS-PAGE. Radiofluorography of the dried gel revealed a specifically labeled protein of 60 kD. The corresponding band from a Coomassie stained gel contained purified QPP.

4. Novelty of QPP Confirmed by Sequencing; Cloning of QPP cDNA

The sequences of four peptides of a QPP tryptic digest are shown in Fig. 1. Peptide GT148 (SEQ ID NO 1) is 13 residues long and has some homology to residues 281-293 of human prolylcarboxypeptidase (PCP). Peptide GT85 (SEQ ID NO 3) is 8 amino acids long and is identical to residues 17-24 of swine dipeptidyl peptidase II (DPPII). Based on these sequences, nucleic acid probes were designed to screen T-cell mRNA samples. The full length mRNA was isolated and sequenced; both the cDNA sequence and its predicted amino acid sequence are shown in Fig. 2 (SEQ ID NOs 5 and 6, respectively).

5. Expression of Recombinant QPP in Quiescent Fibroblasts Increases Cell Survival

The role of QPP in protecting quiescent (non-cycling) cells from death was confirmed in fibroblasts transfected with a QPP transgene sequence using

standard techniques. Expression of the recombinant QPP in serum-starved, G₀ phase fibroblasts inhibited apoptosis, as compared to non-QPP transfected fibroblasts.

Use as a Therapeutic

5 The demonstrated effect of QPP, protecting quiescent cells from apoptotic death, indicates that QPP can be used as a therapeutic to inhibit cell death, not only in T-cells, but also in other non-cycling cell types. For example, non-cycling cells of the central nervous system, such as neurons, will also exhibit a QPP-mediated protective effect against apoptosis. In addition to being non-
10 cycling, neurons exhibit patterns of apoptosis analogous to T-cells, are susceptible to the same killer factors, and have a similar apoptotic signalling cascade. Furthermore, if neurons are denied the required trophic factors and cell-to-cell contact, they die by a mechanism similar to that exhibited in T-cells. All of the above factors are consistent with QPP exerting its effect in neurons as well as T-
15 cells.

QPP-related therapy of non-cycling cells would be useful, for example, when a patient suffers from a condition, or is predisposed to suffer from a condition, associated with inappropriate cell death. For example, given that a number of human neurological disorders, as well as neurological traumatic injury
20 or ischemia, are associated with apoptotic neuronal death, contacting the cells of the nervous system with QPP, or a compound which enhances the QPP effect, will be effective in treating patients suffering from such conditions. In particular, disorders or conditions that may be treated include, without limitation, Alzheimer's disease, Huntington's disease, Parkinson's disease, spinal muscular

dystrophy, ALS, multiple sclerosis, epilepsy, or stroke, as well as spinal cord injury. QPP or an agonist can also be administered therapeutically to patients in need of immune system enhancement, for example, to protect important T-cells such as CD4⁺ cells. Patients which would benefit from this treatment include

5 AIDS patients threatened with reduced CD4⁺ cell counts, and cancer patients with suppressed immune function as a result of their disease, chemotherapy, and/or radiation therapy.

Conversely, inhibitors of QPP can also be used to increase apoptotic death of non-cycling cells. Such therapy may be useful, for example, to kill

10 T-cells, when an immunomodulating drug is needed for the treatment of allograft rejection, graft-versus-host disease, or auto-immune diseases such as rheumatoid arthritis.

Use as a Target for Screening

As briefly noted above, QPP-related therapy can take the form of

15 administering compounds which either activate or inhibit the QPP effect. Such compounds can be identified using QPP as a target in screening assays. Compounds that enhance the QPP effect could act by either increasing QPP enzymatic activity or increasing QPP expression. Compounds that inhibit QPP action, on the other hand, could inhibit QPP enzymatic activity or inhibit QPP

20 expression.

To screen for QPP enzymatic activity, any QPP substrate which contains proline in the penultimate position can be used. For example, the substrate AlaProAFC can be used in a fluorescent cleavage assay. Alternatively, a colorimetric assay can be carried out using as a substrate Gly-Pro-pNA as a

substrate. The choice of terminal amino acid is not critical, provided that the substrate contain a free terminal amino group.

A fluorescence assay can be used, for example, which employs a fluorescence spectrometer for excitation at 400 nm and emission at 505 nm. The 5 spectrometer is calibrated for fluorescence intensity of 0.000=10 mM HEPES, pH 7.4; and fluorescence intensity of 1.000=10 Mm HEPES, 1 M AFC.

To carry out the assay, between 10 and 100 μ l of purified QPP enzyme, is diluted to 1 ml with 10 mM HEPES, pH 7.4, containing 10 mM Ala-Pro-AFC. At least one extract/substrate sample is run without test compound, to provide a 10 standard for comparison with the test sample.

In the test samples, multiple samples are run containing varying concentrations, down to 10^{-8} M, of the test compound. The sample (with or without test compound) is placed in a cuvette, and inserted into a fluorescent spectrometer. Enzymatic activity is measured as the accumulation of fluorescence intensity (i.e., 15 substrate cleavage product) over a period of 1 min. A compound is identified as a QPP modulator if fluorescence is changed from the control value as a result of the presence of the compound.

Candidate compounds which modulate QPP activity *in vitro* can be tested for efficaciousness in secondary cell-based assays. Such assays provide 20 relevant information regarding whether the candidate compound can cross the cell membrane, and produce an intracellular effect.

Cultures of cells are easily prepared for use in apoptosis assays by standard methods known in the art. For example, methods for isolating neurons can be found in Slack, R. S., et al., *J. Cell Biol.*, 135:1085, (1996); Belliveau, D. J., 25 et al., *J. Cell Biol.*, 136:375 (1997); Brewer et al., *Nature* 363:265-266 (1993); or

Henderson et al., *Nature* 363:266-267 (1993). After establishment of a healthy neuron culture, neurons can be induced to undergo apoptosis by exposure to any one of many well-characterized stimuli including NGF or BDNF withdrawal, serum withdrawal, exposure to hypoxic conditions, or the addition of chemicals 5 that induce apoptosis, such as staurosporine or glutamate (see e.g., Koh et al., *Exp. Neurol.*, 135:153-159 (1995); MacManus et al., *Exp. Cell Res.*, 223:310-320, (1997)).

Compounds can also be screened for their effect on QPP gene expression. To screen for such compounds, QPP mRNA levels can be measured in 10 cells which endogenously express QPP, for example, T-cells or neurons, or in cells transfected with QPP's upstream regulators operably linked to either its own QPP coding sequence or a reporter gene. Those compounds identified as increasing or decreasing QPP mRNA levels could increase or decrease the cellular QPP effect.

Proceeding to the next step, candidate compounds that modulate QPP 15 activity or expression in cultured cells can be tested in animal models that are relevant to the disease condition of interest. Under some circumstances, such as the development of treatments for neurodegenerative conditions, the candidate compounds may be further modified to enhance their diffusion or transport across the blood-brain barrier. Particularly useful animal models include, without 20 limitation, Parkinson's disease (PD) rat models, which are established by injecting the catecholamine-specific neurotoxin, 6-hydroxydopamine (6-OHDA), into the medial forebrain bundle or the substantia nigra pars compacta to achieve a rapid degeneration of the nigrostriatal pathway, or into the striatum to achieve progressive degeneration, as has been described (see, for example Gerlach and 25 Riederer, *J. Neural. Transm.* 103: 987-1041, 1996; Bernard et al., *J. Comp.*

Neurol. 368: 553-568, 1996; Asada et al., *Ex. Neurol.* 139: 173-187, 1996).

Alternatively, rats may be rendered "epileptic" (i.e., induced to suffer brain seizures which often result in neuronal cell death) by administration of a variety of compounds including intravenous injection of bicuculline (Blennow et al., *J.*

- 5 *Cereb. Blood Flow Metab.* 5: 439-445, 1995). Furthermore, apoptosis may be induced by axotomy, ischemia, or traumatic insult to induce spinal cord injury, as described by Liu et al. (*J. Neuroscience* 17:5395-5406, 1997).

Test Compounds

In general, drugs for prevention or treatment of a condition related to 10 inappropriate cell death, which function by altering the expression of QPP or its biological activity, are identified from libraries of natural products or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Examples of such extracts or compounds include, but are not limited to, plant-fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic 15 compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds.

Libraries of genomic DNA or cDNA may be generated by standard techniques 20 (see, e.g., Ausubel et al., *supra*) and are also commercially available (Clontech Laboratories Inc., Palo Alto, CA). Nucleic acid libraries used to screen for compounds that alter QPP gene expression or QPP enzymatic activity are not limited to the species from which the QPP gene or protein is derived. For example, a *Xenopus* cDNA may be found to encode a protein that alters human

QPP gene expression or alters human QPP enzymatic activity.

Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI).

Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant,
5 and animal extracts are commercially available from a number of sources,
including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch
Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge,
MA). In addition, natural and synthetically produced libraries are produced, if
desired, according to methods known in the art, e.g., by standard extraction and
10 fractionation methods.

In addition, methods for dereplication (e.g., taxonomic dereplication,
biological dereplication, and chemical dereplication, or any combination thereof)
or the elimination of replicates or repeats of materials already known for their
therapeutic activities for neuroendocrine or pituitary disorders can be employed.

15 When a crude extract is found to modulate QPP's effect, further
fractionation of the positive lead extract is necessary to isolate chemical
constituents responsible. Thus, the goal of the extraction, fractionation, and
purification process is the characterization and identification of a chemical entity
within the crude extract having QPP-modulating activities. The same assays
20 described herein for the detection of activities in mixtures of compounds can be
used to purify the active component and to test derivatives thereof. Methods of
fractionation and purification of such heterogenous extracts are known in the art.
If desired, compounds shown to be useful agents for treatment are chemically
modified according to methods known in the art.

Sources of QPP

QPP can be obtained by purification from human lymphocytes, as described herein, or from other QPP-expressing cells. Alternatively, the enzyme can be produced recombinantly using known techniques. e.g., expression of the
5 QPP coding sequence in mammalian cells such as Chinese hamster ovary cells.

Administration

QPP or the compounds which modulate the QPP effect may be administered by any appropriate route. For example, administration may be parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial,
10 intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration.

Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or
15 aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or
20 hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles,

osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel. The concentration of QPP in the formulation will vary depending upon a number of factors, including the dosage of the drug to be administered, and the route of administration.

The formulations can be administered to human patients in therapeutically effective amounts (e.g., amounts which prevent, eliminate, or 10 reduce the pathological condition) to provide therapy for the disorders described above. Typical dose ranges are from about 0.1 $\mu\text{g}/\text{kg}$ to about 1 g/kg of body weight per day. The preferred dosage of drug to be administered is likely to depend on such variables as the type and extent of the disorder, the overall health status of the particular patient, the formulation of the compound excipients, and its 15 route of administration.

Antibodies Directed Against QPP

The QPP amino acid sequence, or fragments thereof, can be used to generate polyclonal or monoclonal antibodies specific for QPP, using conventional techniques. Such antibodies can be used in the above-described 20 screening assays, and in any of the many known conventional immunoassay formats to measure QPP levels in biological samples, e.g., samples of peripheral blood lymphocytes. A decreased level of lymphocyte QPP, as determined in such assays, is indicative of immune dysfunction in the patient from whom the sample was obtained.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference.

- While the invention has been described in connection with specific
- 5 embodiments, it will be understood that it is capable of further modifications. Therefore, this application is intended to cover any variations, uses, or adaptations of the invention that follow, in general, the principles of the invention, including departures from the present disclosure that come within known or customary practice within the art.
- 10 Other embodiments are within the claims.

What is claimed is:

Claims

1. A substantially pure, recombinant quiescent cell post-prolyl dipeptidase polypeptide (QPP), wherein said polypeptide inhibits quiescent cells from undergoing apoptosis.
- 5 2. The polypeptide of claim 1, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO 6.
3. A substantially pure nucleic acid molecule encoding a QPP polypeptide, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO 6.
- 10 4. A substantially pure nucleic acid molecule encoding a QPP polypeptide, wherein said molecule comprises SEQ ID NO 5 or a sequence that is complementary to a sequence that hybridizes to SEQ ID NO 5 under stringent hybridization conditions.
5. A host cell transfected with a transgene comprising a nucleic acid molecule encoding a QPP polypeptide, wherein said molecule comprises SEQ ID NO 5 or a sequence that is complementary to a sequence that hybridizes to SEQ ID NO 5 under stringent hybridization conditions, or a sequence that encodes a QPP polypeptide of SEQ ID NO 6, wherein said nucleic acid molecule is operably linked to a regulatory sequence.

6. A method of producing a QPP polypeptide comprising:
 - providing a cell transformed or transfected with a nucleic acid molecule encoding a QPP polypeptide operably linked to a regulatory sequence, wherein said molecule comprises SEQ ID NO 5, or a sequence that is complementary to a sequence that hybridizes to SEQ ID NO 5 under stringent hybridization conditions, or a sequence that encodes a QPP polypeptide of SEQ ID NO 6;
 - 5 culturing said cell under conditions for expressing said nucleic acid; and
 - isolating said QPP polypeptide.
7. A method of identifying a therapeutic candidate compound that
10 modulates a QPP effect in quiescent cells, said method comprising contacting a recombinant QPP polypeptide, or a host cell of claim 5, with said compound and measuring QPP biological activity.
8. A method of inhibiting apoptosis in a quiescent cell, said method comprising contacting said cell with a recombinant QPP polypeptide in an amount
15 sufficient to protect said cell from death when an apoptosis stimulus is administered.
9. A method of treating a mammal for a condition related to quiescent cell death, said method comprising administering to said mammal a recombinant QPP polypeptide in an amount sufficient to inhibit said cell death.
- 20 10. The method of claim 9, wherein said condition is a neurodegenerative disorder.

11. The method of claim 9, wherein said condition is an immune system disorder.

12. The method of claim 7, 8, or 9, wherein said QPP polypeptide comprises the amino acid sequence of SEQ ID NO: 6.

5 13. The method of claim 9, wherein administering said QPP polypeptide comprises transplanting a host cell into said mammal, said host cell *ex vivo* transfected with a transgene comprising a nucleic acid molecule encoding a QPP polypeptide, wherein said nucleic acid molecule is operably linked to a regulatory sequence and is expressed in said transplanted cell.

FIGURE 1

Asn Ala Phe Thr Val Leu Ala Met Met Asp Tyr Pro Tyr (SEQ ID NO 1)

Asp Leu Phe Leu Gln Gly Ala Tyr Asp Thr Val Arg (SEQ ID NO 2)

Leu Asp His Phe Asn Phe Glu Arg (SEQ ID NO 3)

Asp Val Thr Ala Asp Phe Glu Gly Gln Ser Pro Lys (SEQ ID NO 4)

FIGURE 2
(Page 1 of 3)
nucleic acid (SEQ ID NO 5); amino acid (SEQ ID NO 6)

atg ggc tcc gct ccc tgg gcc ccg gtc ctg ctg ctg gcg ctc ggg ctg		48
Met Gly Ser Ala Pro Trp Ala Pro Val Leu Leu Leu Ala Leu Gly Leu		
1	5	10
		15
cgc ggc ctc cag gcg ggg gcc ccg agg gcc ccg gac ccc ggc ttc cag		96
Arg Gly Leu Gln Ala Gly Ala Arg Arg Ala Pro Asp Pro Gly Phe Gln		
20	25	30
gag cgc ttc ttc cag cag cgt ctg gac cac ttc gag cgc ttc		144
Glu Arg Phe Phe Gln Gln Arg Leu Asp His Phe Asn Phe Glu Arg Phe		
35	40	45
ggc aac aag acc ttc ccc cag cgc ttc ctg gtg tcg gac agg ttc tgg		192
Gly Asn Lys Thr Phe Pro Gln Arg Phe Leu Val Ser Asp Arg Phe Trp		
50	55	60
gtc cgg ggc gag ggg ccc acc ttc ttc tac act ggg aac gag ggc gac		240
Val Arg Gly Glu Gly Pro Thr Phe Phe Tyr Thr Gly Asn Glu Gly Asp		
65	70	75
		80
gtg tgg gcc ttc gcc aac aac tcg ggc ttc gtc gcg gag ctg gcg gcc		288
Val Trp Ala Phe Ala Asn Asn Ser Gly Phe Val Ala Glu Leu Ala Ala		
85	90	95
gag cgg ggg gct cta ctg gtc ttc gcg gag cac cgc tac tac ggg aag		336
Glu Arg Gly Ala Leu Leu Val Phe Ala Glu His Arg Tyr Tyr Gly Lys		
100	105	110
tcc ctg ccg ttc ggt gcg cag tcc acg cag cgc ggg cac acg gag ctg		384
Ser Leu Pro Phe Gly Ala Gln Ser Thr Gln Arg Gly His Thr Glu Leu		
115	120	125
ctg acg gtg gag cag gcc ctg gcc gac ttc gca gag ctg ctc cgc gcg		432
Leu Thr Val Glu Gln Ala Leu Ala Asp Phe Ala Glu Leu Leu Arg Ala		
130	135	140
cta cga cgc gac ctc ggg gcc cag gat gcc ccc gcc atc gcc ttc ggt		480
Leu Arg Arg Asp Leu Gly Ala Gln Asp Ala Pro Ala Ile Ala Phe Gly		
145	150	155
		160
gga agt tat ggg ggg atg ctc agt gcc tac ctg agg atg aag tat ccc		528
Gly Ser Tyr Gly Gly Met Leu Ser Ala Tyr Leu Arg Met Lys Tyr Pro		
165	170	175
cac ctg gtg gcg ggg gcc ctg gcg gcc agc gcg ccc gtt cta gct gtg		576
His Leu Val Ala Gly Ala Leu Ala Ala Ser Ala Pro Val Leu Ala Val		
180	185	190

FIGURE 2
(Page 2 of 3)

gca ggc ctc ggc gac tcc aac cag ttc ttc cggtt gac gtc acg ggc gac Ala Gly Leu Gly Asp Ser Asn Gln Phe Phe Arg Asp Val Thr Ala Asp	624
195 200 205	
 ttt gag ggc cag agt ccc aaa tgc acc cag ggt gtgg cggtt gaa ggc ttc Phe Glu Gly Gln Ser Pro Lys Cys Thr Gln Gly Val Arg Glu Ala Phe	672
210 215 220	
 cga cag atc aag gac ttgtt cta cag gga gcc tac gac acg gtc cgc Arg Gln Ile Lys Asp Leu Phe Leu Gln Gly Ala Tyr Asp Thr Val Arg	720
225 230 235 240	
 tgg gag ttc ggc acc tgc cag ccgtt ctgtt cttt gac gag aag gac ctgtt acc Trp Glu Phe Gly Thr Cys Gln Pro Leu Ser Asp Glu Lys Asp Leu Thr	768
245 250 255	
 cag ctc ttc atg ttc gcc cgg aat gttt acc gttt ctgtt gcc atgtt atgtt Gln Leu Phe Met Phe Ala Arg Asn Ala Phe Thr Val Leu Ala Met Met	816
260 265 270	
 gac tac ccc tac ccc act gac ttc ctgtt ggtt ccc ctc cctt gcc aac ccc Asp Tyr Pro Tyr Pro Thr Asp Phe Leu Gly Pro Leu Pro Ala Asn Pro	864
275 280 285	
 gtc aag gtgtt ggc tgtt gat cgg ctgtt agttt gag ggc cttt cttt agg atctt acgtt Val Lys Val Gly Cys Asp Arg Leu Leu Ser Glu Ala Gln Arg Ile Thr	912
290 295 300	
 ggg ctgtt cgtt gca ctgtt gca ggg ctgtt gtc tac aac gttt gcc tcgtt ggc tcc gag Gly Leu Arg Ala Leu Ala Gly Leu Val Tyr Asn Ala Ser Gly Ser Glu	960
305 310 315 320	
 cac tgc tac gac atc tac cgg ctc tac cac agc tgtt gct gac ccc act His Cys Tyr Asp Ile Tyr Arg Leu Tyr His Ser Cys Ala Asp Pro Thr	1008
325 330 335	
 ggc tgc ggc acc ggc ccc gac gttt gcc agg gttt tgg gac tac cag ggc tgc Gly Cys Gly Thr Gly Pro Asp Ala Arg Ala Trp Asp Tyr Gln Ala Cys	1056
340 345 350	
 acc gag atc aac ctgtt acc ttc gttt gcc agc aac aat gtgtt acc gat atgtt ttc Thr Glu Ile Asn Leu Thr Phe Ala Ser Asn Asn Val Thr Asp Met Phe	1104
355 360 365	
 ccg gac ctgtt ccc ttc act gac gag ctc cgc cag cgg tac tgc ctgtt gac Pro Asp Leu Pro Phe Thr Asp Glu Leu Arg Gln Arg Tyr Cys Leu Asp	1152
370 375 380	

FIGURE 2
(Page 3 of 3)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/24052

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 9/48, 9/50, 5/02; C07H 21/04; C07K 14/00; A61K 38/43
 US CL : 435/183, 212, 219, 325, 4; 530/350; 536/23.5; 514/2, 12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/183, 212, 219, 325, 4; 530/350; 536/23.5; 514/2, 12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, BIOSIS, CAPLUS
 search terms: post-prolyl dipeptidase, peptidase, dipeptidyl, apoptosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	US 6,040,145 A (HUBER et al) 21 March 2000, abstract and column 1, lines 28-52.	1
X,P	Database SPTREMBL on STN, Accession No. Q9UHL4, "Sequence, purification, and cloning of an intracellular serine protease, quiescent cell proline dipeptidase", J. Biol. Chem., 274: 34053-34058, 1999.	1-4

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
18 DECEMBER 2000

Date of mailing of the international search report

22 JAN 2001

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